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(54) Title: MUTEINS OF FIBROBLAST GROWTH FACTOR 21

(57) Abstract: The present invention relates to novel muteins of human fibroblast growth factor 21 with improved pharmaceutical properties. Both protein and the respective encoding nucleic acid species are disclosed. The invention also embodies vectors and host cells for the propagation of said nucleic acid sequences and the production of said muteins. Also disclosed are methods for treating type 2 diabetes, obesity, metabolic syndrome, and in reducing the mortality and morbidity of critically ill patients.

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5                   **MUTEINS OF FIBROBLAST GROWTH FACTOR 21****BACKGROUND OF THE INVENTION****Field of the Invention**

10                   The present invention relates to the identification of new muteins of fibroblast growth factor 21 that have improved pharmaceutical properties.

**Description of the Related Art**

15                   Fibroblast growth factors are large polypeptides widely expressed in developing and adult tissues (Baird et al., *Cancer Cells*, 3:239-243, 1991) and play crucial roles in multiple physiological functions including angiogenesis, mitogenesis, pattern formation, cellular differentiation, metabolic regulation and repair of tissue injury (McKeehan et al.,  
20                   *Prog. Nucleic Acid Res. Mol. Biol.* 59:135-176, 1998). According to the published literature, the FGF family now consists of at least twenty-three members, FGF-1 to FGF-23 (Reuss et al., *Cell Tissue Res.* 313:139-157 (2003).

                  Fibroblast growth factor 21 (FGF-21) has been reported to be preferentially expressed in the liver (Nishimura et al., *Biochimica et Biophysica Acta*, 1492:203-206,  
25                   (2000); WO01/36640; and WO01/18172) and described as a treatment for ischemic vascular disease, wound healing, and diseases associated with loss of pulmonary, bronchia or alveolar cell function and numerous other disorders. More recently, FGF-21 has been shown to stimulate glucose-uptake in mouse 3T3-L1 adipocytes in the presence and absence of insulin, and to decrease fed and fasting blood glucose, triglycerides, and  
30                   glucagon levels in *ob/ob* and *db/db* mice and 8 week old ZDF rats in a dose-dependant manner, thus, providing the basis for the use of FGF-21 as a therapy for treating diabetes and obesity (WO03/011213). In addition, FGF-21 has been shown to be effective in reducing the mortality and morbidity of critically ill patients (WO03/059270).

                  A significant challenge in the development of protein pharmaceuticals, such as  
35                   FGF-21, is to cope with their physical and chemical instabilities. The compositional variety and characteristics of proteins define specific behaviors such as folding, conformational stability, and unfolding/denaturation. Such characteristics must be

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5 addressed to stabilize proteins when developing pharmaceutical formulation conditions utilizing aqueous protein solutions (Wang, W., *Int. J. of Pharmaceutics*, 18, (1999).

Specifically, in pharmaceutical protein development, anti-microbial preservative agents such as phenol, *m*-cresol, methylparaben, resorcinol, and benzyl alcohol are necessary in parenteral pharmaceutical formulations that are intended to be a sterile,  
10 multi-use formulation. Unfortunately, these compounds often adversely affect the stability of the protein product, triggering association and aggregation, in particular (Maa *et al.*, *Int. J. of Pharmaceutics* 140:155-168 (1996); Lam *et al.*, *Pharm. Res.* 14(6):725-729 (1997)).

FGF-21 will likely be utilized as a multi-use, sterile pharmaceutical formulation.  
15 However, it has been determined that preservatives, *i.e.* *m*-cresol, have an adverse affect on its stability under these conditions. Clearly, there is a need to develop a stable aqueous protein formulation for the therapeutic protein FGF-21. The present invention overcomes the significant hurdles of physical instabilities with the invention of muteins of FGF-21 that are more stable than wild-type FGF-21 under pharmaceutical formulation conditions.  
20 Thus, the muteins of FGF-21 of the present invention provide stable pharmacological protein formulations that are useful for the treatment of type 2 diabetes, obesity, metabolic syndrome, and in reducing the mortality and morbidity of critically ill patients.

#### Summary of the Invention

25 In a first aspect, the present invention provides muteins of human fibroblast growth factor 21, or a biologically active peptide thereof, comprising the substitution with a charged and/or polar but uncharged amino acid for one or more of the following: glycine 42, glutamine 54, arginine 77, alanine 81, leucine 86, phenylalanine 88, lysine 122, histidine 125, arginine 126, proline 130, arginine 131, leucine 139, alanine 145,  
30 leucine 146, isoleucine 152, alanine 154, glutamine 156, glycine 161, serine 163, glycine 170, or serine 172 wherein the numbering of the amino acids is based on SEQ ID NO:1.

A second aspect of the present invention provides muteins of human fibroblast growth factor 21, or a biologically active peptide thereof, comprising the substitution of a cysteine for two or more of the following: arginine 19, tyrosine 20, leucine 21, tyrosine  
35 22, threonine 23, aspartate 24, aspartate 25, alanine 26, glutamine 27, lutamine 28, alanine 31, leucine 33, isoleucine 35, leucine 37, valine 41, glycine 42, glycine 43,

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5 glutamate 50, glutamine 54, leucine 58, valine 62, leucine 66, glycine 67, lysine 69,  
arginine 72, phenylalanine 73, glutamine 76, arginine 77, aspartate 79, glycine 80, alanine  
81, leucine 82, glycine 84, serine 85, proline 90, alanine 92, serine 94, phenylalanine 95,  
leucine 100, aspartate 102, tyrosine 104, tyrosine 107, serine 109, glutamate 110, proline  
115, histidine 117, leucine 118, proline 119, asparagine 121, lysine 122, serine 123,  
10 proline 124, histidine 125, arginine 126, aspartate 127, alanine 129, proline 130, glycine  
132, alanine 134, arginine 135, leucine 137, proline 138, or leucine 139, wherein the  
numbering of the amino acids is based on SEQ ID NO:1.

A third aspect of the present invention provides muteins of human FGF-21, or a  
biologically active peptide thereof, comprising the substitution with any charged and/or  
15 polar but uncharged amino acid at any of the amino acid positions indicated in the first  
embodiment of the present invention in combination with the substitution of a cysteine at  
two or more amino acid positions indicated in the second embodiment of the invention.

Other embodiments are drawn to polynucleotides encoding the muteins of the  
first, second, and third embodiments, a vector containing said polynucleotides and a host  
20 cell carrying said vector. Another embodiment is drawn to processes to produce a  
polypeptide, to produce cells capable of producing said polypeptide and to produce a  
vector containing DNA encoding said polypeptide.

Yet another embodiment is drawn to methods of treating a patient exhibiting one  
or more of obesity, type 2 diabetes, insulin resistance, hyperinsulinemia, glucose  
25 intolerance, hyperglycemia, or metabolic syndrome comprising administering to said  
patient in need of such treatment a therapeutically effective amount of a human FGF-21  
mucin of the first, second, or third embodiment or a pharmaceutical composition thereof.

#### Detailed Description of the Invention

30 For purposes of the present invention, as disclosed and claimed herein, the  
following terms are as defined below.

FGF-21 is a 208 amino acid polypeptide containing a 27 amino acid leader  
sequence. Human FGF-21 has ~79% amino acid identity to mouse FGF-21 and ~80%  
amino acid identity to rat FGF-21. Human FGF-21 is the preferred polypeptide template  
35 for the muteins of the present invention but it is recognized that one with skill in the art

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- 5 could readily make muteins based on an alternative mammalian FGF-21 polypeptide sequence.

The amino acid positions of the muteins of the present invention are determined from the mature human 181 amino acid FGF-21 polypeptide as shown below (SEQ ID NO:1):

10 1 10 20  
 His Pro Ile Pro Asp Ser Ser Pro Leu Leu Gln Phe Gly Gly Gln Val Arg Gln Arg Tyr  
 30 40  
 Leu Tyr Thr Asp Asp Ala Gln Gln Thr Glu Ala His Leu Glu Ile Arg Glu Asp Gly Thr  
 15 50 60  
 Val Gly Gly Ala Ala Asp Gln Ser Pro Glu Ser Leu Leu Gln Leu Lys Ala Leu Lys Pro  
 70 80  
 Gly Val Ile Gln Ile Leu Gly Val Lys Thr Ser Arg Phe Leu Cys Gln Arg Pro Asp Gly  
 90 100  
 20 Ala Leu Tyr Gly Ser Leu His Phe Asp Pro Glu Ala Cys Ser Phe Arg Glu Leu Leu Leu  
 110 120  
 Glu Asp Gly Tyr Asn Val Tyr Gln Ser Glu Ala His Gly Leu Pro Leu His Leu Pro Gly  
 130 140  
 Asn Lys Ser Pro His Arg Asp Pro Ala Pro Arg Gly Pro Ala Arg Phe Leu Pro Leu Pro  
 150 160  
 25 Gly Leu Pro Pro Ala Leu Pro Glu Pro Pro Gly Ile Leu Ala Pro Gln Pro Pro Asp Val  
 170 180  
 Gly Ser Ser Asp Pro Leu Ser Met Val Gly Pro Ser Gln Gly Arg Ser Pro Ser Tyr Ala  
 30 Ser

The corresponding DNA sequence coding for the mature human 181 amino acid FGF-21 polypeptide is (SEQ ID NO:2):

35 CACCCCATCCCTGACTCCAGTCCTCTCCTGCAATTCGGGGGCCAAGTCCGGCA  
 GCGGTACCTCTACACAGATGATGCCAGCAGACAGAAGCCCACCTGGAGATC  
 AGGGAGGATGGGACGGTGGGGGGCGCTGCTGACCAGAGCCCCGAAAGTCTC  
 CTGCAGCTGAAAGCCTTGAAGCCGGGAGTTATTCAAATCTTGGGAGTCAAGA  
 CATCCAGGTTCTGTGCCAGCGGCCAGATGGGGCCCTGTATGGATCGCTCCAC  
 TTTGACCCTGAGGCCTGCAGCTTCCGGGAGCTGCTTCTTGAGGACGGATACAA

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5 TGTTTACCAAGTCCGAAGCCACGGCCTCCCGCTGCACCTGCCAGGGAACAAG  
TCCCCACACCGGGACCCTGCACCCCGAGGACCAGCTCGCTTCCTGCCACTACC  
AGGCCTGCCCCCGCACTCCCGGAGCCACCCGGAATCCTGGCCCCCAGCCC  
CCCGATGTGGGCTCCTCGGACCCTCTGAGCATGGTGGGACCTTCCCAGGGCCG  
AAGCCCCAGCTACGCTTCC

10 One skilled in the art of expression of proteins will recognize that methionine or methionine-arginine sequence can be introduced at the N-terminus of the mature sequence (SEQ ID NO: 1) for expression in *E. coli* and are contemplated within the context of this invention.

Amino acids are identified using the three-letter code or alternatively could be  
15 designated using the standard one letter code. Mutations are designated by the three-letter code for the original amino acid, followed by the amino acid number, followed by the three-letter code for the replacement amino acid. The numerical designations of each mutein is based on the 181 amino acid sequence of mature, wild-type, human FGF-21. For example, a substitution for leucine at position 139 (*i.e.* Leu139) with the negatively  
20 charged amino acid, glutamate (Glu) is designated as Leu139Glu or L139E. In a similar fashion, the double substitution for isoleucine at position 152 and serine at position 163 (Ile152, Ser163) with the negatively charged amino acid, glutamate (Glu) is designated as Ile152Glu/Ser163Glu, I152E/S163E or I152E-S163E.

A human FGF-21 mutein is defined as comprising human FGF-21 in which at  
25 least one amino acid of the wild-type mature protein has been substituted by another amino acid. Generally speaking, a mutein possesses some modified property, structural or functional, of the wild-type protein. For example, the mutein may have enhanced or improved physical stability in concentrated solutions (*e.g.*, less hydrophobic mediated aggregation), while maintaining a favorable bioactivity profile. The mutein may possess  
30 increased compatibility with pharmaceutical preservatives (*e.g.*, *m*-cresol, phenol, benzyl alcohol), thus enabling the preparation of a preserved pharmaceutical formulation that maintains the physiochemical properties and biological activity of the protein during storage. Accordingly, muteins with enhanced pharmaceutical stability when compared to wild-type FGF-21, have improved physical stability in concentrated solutions under both  
35 physiological and preserved pharmaceutical formulation conditions, while maintaining

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5 biological potency. As used herein, these terms are not limiting, it being entirely possible that a given mutein has one or more modified properties of the wild-type protein.

A “biologically active peptide” is defined as a peptide of a mutein of the present invention that maintains the modified property(s) and the biological potency of the mutein.

A “therapeutically-effective amount” is the minimal amount of an active agent  
10 necessary to impart therapeutic benefit to a patient. For example, a “therapeutically-effective amount” to a patient exhibiting, suffering or prone to suffer or to prevent it from suffering from type 2 diabetes, obesity, or metabolic syndrome is such an amount which induces, ameliorates or otherwise causes an improvement in the pathological symptoms, disease progression, physiological conditions associated with or resistance to succumbing  
15 to the afore mentioned disorders. For the purposes of the present invention a “subject” or “patient” is preferably a human, but can also be an animal, more specifically, a companion animal (*e.g.*, dogs, cats, and the like), farm animals (*e.g.*, cows, sheep, pigs, horses, and the like) and laboratory animals (*e.g.*, rats, mice, guinea pigs, and the like).

“Type 2 diabetes” is characterized by excess glucose production in spite of the  
20 availability of insulin, and circulating glucose levels remain excessively high as a result of inadequate glucose clearance.

“Glucose intolerance” can be defined as an exceptional sensitivity to glucose.

“Hyperglycemia” is defined as an excess of sugar (glucose) in the blood.

“Hypoglycemia”, also called low blood sugar, occurs when your blood glucose  
25 level drops too low to provide enough energy for your body’s activities.

“Hyperinsulinemia” is defined as a higher-than-normal level of insulin in the blood.

“Insulin resistance” is defined as a state in which a normal amount of insulin produces a subnormal biologic response.

30 “Obesity”, in terms of the human subject, can be defined as that body weight over 20 percent above the ideal body weight for a given population (R.H. Williams, Textbook of Endocrinology, 1974, p.904-916).

“Metabolic syndrome” can be defined as a cluster of at least three of the following signs: abdominal fat — in most men, a 40-inch waist or greater; high blood sugar — at least  
35 110 milligrams per deciliter (mg/dl) after fasting; high triglycerides — at least 150 mg/dL in the bloodstream; low HDL — less than 40 mg/dl; and, blood pressure of 130/85 or higher.

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5           The critically ill patients encompassed by the present invention generally experience an unstable hypermetabolic state. This unstable metabolic state is due to changes in substrate metabolism, which may lead to relative deficiencies in some nutrients. Generally there is an increased oxidation of both fat and muscle.

10           Moreover, critically ill patients are preferably patients that experience systemic inflammatory response syndrome or respiratory distress. A reduction in morbidity means reducing the likelihood that a critically ill patient will develop additional illnesses, conditions, or symptoms or reducing the severity of additional illnesses, conditions, or symptoms. For example reducing morbidity may correspond to a decrease in the incidence of bacteremia or sepsis or complications associated with multiple organ failure.

15           "Systemic inflammatory response syndrome (SIRS)" as used herein describes an inflammatory process associated with a large number of clinical conditions and includes, but is not limited to, more than one of the following clinical manifestations: (1) a body temperature greater than 38°C or less than 36°C; (2) a heart rate greater than 90 beats per minute; (3) tachypnea, manifested by a respiratory rate greater than 20 breaths per  
20           minute, or hyperventilation, as indicated by a PaCO<sub>2</sub> of less than 32 mm Hg; and (4) an alteration in the white blood cell count, such as a count greater than 12,000/cu mm, a count less than 4,000/cu mm, or the presence of more than 10% immature neutrophils. These physiologic changes should represent an acute alteration from baseline in the absence of other known causes for such abnormalities, such as chemotherapy, induced  
25           neutropenia, and leukopenia.

          "Sepsis" as used herein is defined as a SIRS arising from infection. Noninfectious pathogenic causes of SIRS may include pancreatitis, ischemia, multiple trauma and tissue injury, *i.e.* crushing injuries or severe burns, hemorrhagic shock, immune-mediated organ injury, and the exogenous administration of such putative mediators of the inflammatory  
30           process as tumor necrosis factor and other cytokines.

          Septic shock and multi-organ dysfunction are major contributors to morbidity and mortality in the ICU setting. Sepsis is associated with and mediated by the activation of a number of host defense mechanisms including the cytokine network, leukocytes, and the complement cascade, and coagulation/fibrinolysis systems including the endothelium.

35           Disseminated intravascular coagulation (DIC) and other degrees of consumption coagulopathy associated with fibrin deposition within the microvasculature of various



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5 organs are manifestations of sepsis/septic shock. The downstream effects of the host defense response on target organs is an important mediator in the development of the multiple organ dysfunction syndrome (MODS) and contributes to the poor prognosis of patients with sepsis, severe sepsis, and sepsis complicated by shock.

10 "Respiratory distress" as used herein denotes a condition wherein patients have difficulty breathing due to some type of pulmonary dysfunction. Often these patients exhibit varying degrees of hypoxemia that may or may not be refractory to treatment with supplemental oxygen.

15 Respiratory distress may occur in patients with impaired pulmonary function due to direct lung injury or may occur due to indirect lung injury such as in the setting of a systemic process. In addition, the presence of multiple predisposing disorders substantially increases the risk, as does the presence of secondary factors such as chronic alcohol abuse, chronic lung disease, and a low serum pH.

20 Some causes of direct lung injury include pneumonia, aspiration of gastric contents, pulmonary contusion, fat emboli, near drowning, inhalation injury, high altitude and reperfusion pulmonary edema after lung transplantation or pulmonary embolectomy. Some causes of indirect lung injury include sepsis, severe trauma with shock and multiple transfusions, cardiopulmonary bypass, drug overdose, acute pancreatitis, and transfusions of blood products.

25 One class of pulmonary disorders that causes respiratory distress are associated with the syndrome known as Cor Pulmonale. These disorders are associated with chronic hypoxemia resulting in raised pressure within the pulmonary circulation called pulmonary hypertension. The ensuing pulmonary hypertension increases the work-load of the right ventricle, thus leading to its enlargement or hypertrophy. Cor Pulmonale generally presents as right heart failure defined by a sustained increase in right ventricular pressures and clinical evidence of reduced venous return to the right heart.

30 "Chronic obstructive pulmonary diseases" (COPDs), which include emphysema and chronic bronchitis also cause respiratory distress and are characterized by obstruction to air flow. COPDs are the fourth leading cause of death and claim over 100,000 lives annually.

35 "Acute respiratory distress syndrome" (ARDS) is generally progressive and characterized by distinct stages. The syndrome is generally manifested by the rapid onset

5 of respiratory failure in a patient with a risk factor for the condition. Arterial hypoxemia that is refractory to treatment with supplemental oxygen is a characteristic feature. There may be alveolar filling, consolidation, and atelectasis occurring in dependent lung zones; however, non-dependent areas may have substantial inflammation. The syndrome may progress to fibrosing alveolitis with persistent hypoxemia, increased alveolar dead space, and a further decrease in pulmonary compliance. Pulmonary hypertension, which results from damage to the pulmonary capillary bed, may also develop.

The first preferred aspect of the invention comprises muteins of human FGF-21 in which substitution means that any charged and/or polar but uncharged amino acid replaces at least one of the following: glycine 42, glutamine 54, arginine 77, alanine 81, leucine 86, phenylalanine 88, lysine 122, histidine 125, arginine 126, proline 130, arginine 131, leucine 139, alanine 145, leucine 146, isoleucine 152, alanine 154, glutamine 156, glycine 161, serine 163, glycine 170, or serine 172, wherein the numbering of the amino acids is based on SEQ ID NO:1. A charged amino acid is defined as a positively or negatively charged amino acid. A positively charged amino acid is defined to include histidine, lysine, arginine, and non-naturally occurring analogs thereof (*e.g.*, gamma aminobutyric acid, ornithine, *etc.*). A negatively charged amino acid is defined to include aspartate, glutamate, and non-naturally occurring analogs thereof (*e.g.*, aminoadipic acid). A polar but uncharged amino acid is defined to include serine, threonine, asparagine, glutamine, and non-naturally occurring analogs thereof. The most preferred muteins of the first embodiment are Gln54Glu, Leu139Glu, Ala145Glu, Leu146Glu, Ile152Glu, Gln156Glu, Ser163Glu, and Ile152Glu-Ser163Glu.

The second aspect of the present invention provides muteins of human FGF-21, or a biologically active peptide thereof, comprising the substitution of a cysteine for two or more of the following: arginine 19, tyrosine 20, leucine 21, tyrosine 22, threonine 23, aspartate 24, aspartate 25, alanine 26, glutamine 27, leucine 28, alanine 31, leucine 33, isoleucine 35, leucine 37, valine 41, glycine 42, glycine 43, glutamate 50, glutamine 54, leucine 58, valine 62, leucine 66, glycine 67, lysine 69, arginine 72, phenylalanine 73, glutamine 76, arginine 77, aspartate 79, glycine 80, alanine 81, leucine 82, glycine 84, serine 85, proline 90, alanine 92, serine 94, phenylalanine 95, leucine 100, aspartate 102, tyrosine 104, tyrosine 107, serine 109, glutamate 110, proline 115, histidine 117, leucine 118, proline 119, asparagine 121, lysine 122, serine 123, proline 124, histidine 125,

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5 arginine 126, aspartate 127, alanine 129, proline 130, glycine 132, alanine 134, arginine 135, leucine 137, proline 138, or leucine 139, wherein the numbering of the amino acids is based on SEQ ID NO:1.

One skilled in the art will also recognize that the native cysteines, cysteine 75 and cysteine 93, could also be utilized as loci to introduce a novel disulfide bond that may  
10 impart improved properties. Specifically contemplated is the introduction of a cysteine substitution at serine 85 or phenylalanine 73, coupled with a concomitant change at either cysteine 93 or cysteine 75, respectively, wherein the latter sites are replaced with any other amino acid.

Naturally occurring disulfide bonds, as provided by cysteine residues, generally  
15 increase thermodynamic stability of proteins. Successful examples of increased thermodynamic stability, as measured in increase of the melting temperature, are multiple disulfide-bonded mutants of the enzymes T4 lysozyme (Matsumura, *et al.*, *PNAS* 86:6562-6566 (1989)) and barnase (Johnson *et al.*, *J. Mol. Biol.* 268:198-208 (1997)). An aspect of the present invention is the premise that constraining the flexibility of the 118-  
20 134 amino acid loop of FGF-21 by disulfide bonds enhances the physical stability of FGF-21 in the presence of a preservative, presumably by limiting access of the preservative to the hydrophobic core of the protein.

Mutins of FGF-21 with engineered disulfide bonds, in addition to the naturally occurring one at Cys75-Cys93, are as follows: Gln76Cys-Ser109Cys, Cys75-Ser85Cys,  
25 Cys75-Ala92Cys, Phe73Cys-Cys93, Ser123Cys-His125-Cys, Asp102Cys-Tyr104Cys, Asp127Cys-Gly132Cys, Ser94Cys-Glu110Cys, Pro115Cys-His117Cys, Asn121Cys-Asp127Cys, Leu100Cys-Asp102Cys, Phe95Cys-Tyr107Cys, Arg19Cys-Pro138Cys, Tyr20Cys-Leu139Cys, Tyr22Cys-Leu137Cys, Arg77Cys-Asp79Cys, Pro90Cys-Ala92Cys, Glu50Cys-Lys69Cys, Thr23Cys-Asp25Cys, Ala31Cys-Gly43Cys, Gln28Cys-Gly43Cys, Thr23Cys-Gln28Cys, Val41Cys-Leu82Cys, Leu58Cys-Val62Cys, Gln54Cys-Leu66Cys, Ile35Cys-Gly67Cys, Gly67Cys-Arg72Cys, Ile35Cys-Gly84Cys, Arg72Cys-Gly84Cys, or Arg77Cys-Ala81Cys, wherein the numbering of the amino acids is based  
30 on SEQ ID NO:1. Preferred mutins with engineered disulfide bonds are Tyr22Cys-Leu139Cys; Asp24Cys-Arg135Cys; Leu118Cys-Gly132Cys; His117Cys-Pro130Cys; His117Cys-Ala129Cys; Leu82Cys-Pro119Cys; Gly80Cys-Ala129Cys; Gly43Cys-Pro124Cys; Gly42Cys-Arg126Cys; Gly42Cys-Pro124Cys; Gln28Cys-Pro124Cys;  
35

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5 Gln27Cys-Ser123Cys; Ala26Cys-Lys122Cys; or Asp25Cys-Lys122Cys. Most preferred  
mutedins with engineered disulfide bonds are Leu118Cys-Ala134Cys; Leu21Cys-  
Leu33Cys; Ala26Cys-Lys122Cys; Leu21Cys-Leu33Cys/Leu118Cys-Ala134Cys

The third aspect of the present invention provides mutedins of human FGF-21, or a  
biologically active peptide thereof, comprising a substitution of any charged and/or polar  
10 but uncharged amino acid at any of the amino acid positions indicated in the first  
embodiment of the present invention combined with the substitution of a cysteine at two  
or more amino acid positions indicated in the second embodiment of the invention.

It is well known in the art that a significant challenge in the development of  
protein pharmaceuticals is to deal with the physical and chemical instabilities of proteins.  
15 This is even more apparent when a protein pharmaceutical formulation is intended to be a  
multiple use, injectable formulation requiring a stable, concentrated and preserved  
solution, while maintaining a favorable bioactivity profile. Detailed biophysical  
characterization of wild-type FGF-21 established that a concentrated protein solution (> 5  
mg/ml), when exposed to stress conditions, such as high temperature or low pH, lead to  
20 accelerated association and aggregation (*i.e.*, poor physical stability and  
biopharmaceutical properties). Exposure of a concentrated protein solution of FGF-21 to  
pharmaceutical preservatives (*e.g.*, *m*-cresol) also had a negative impact on physical  
stability.

Therefore, an embodiment of the present invention is to enhance physical stability  
25 of concentrated solutions, while maintaining chemical stability and biological potency,  
under both physiological and preserved formulation conditions. It is thought that  
association and aggregation may result from hydrophobic interactions, since, at a given  
protein concentration, temperature, and ionic strength have considerable impact on  
physical stability. For the most part, non-conserved, presumed surface exposed amino  
30 acid residues were targeted. The local environment of these residues was analyzed and,  
those that were not deemed structurally important were selected for mutagenesis. One  
method to initiate specific changes is to further decrease the pI of the protein by  
introducing glutamic acid residues ("glutamic acid scan"). It is hypothesized that the  
introduction of charged substitutes would inhibit hydrophobic-mediated aggregation via  
35 charge-charge repulsion and potentially improve preservative compatibility. In addition,  
one skilled in the art would also recognize that with sufficient degree of mutagenesis the

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5 *pI* could be shifted into a basic pH range by the introduction of positive charge with or without concomitant decrease in negative charge, thus allowing for charge-charge repulsion.

Although the embodiments of the present invention concern the physical and chemical stability under both physiological and preserved pharmaceutical formulation  
10 conditions, maintaining the biological potency of the muteins as compared to wild-type FGF-21 is an important factor of consideration as well. Therefore, the biological potency of the muteins of the present invention is defined by the ability of the muteins to affect glucose uptake as measured in the *in vitro* 3T3-L1 cell assay (Example 4) and/or the lowering of plasma glucose levels, as well as, plasma triglycerides, as measured *in vivo* in  
15 the *ob/ob* mouse assay (Example 5).

The muteins of FGF-21 administered according to this invention may be generated and/or isolated by any means known in the art. The most preferred method for producing the mutein is through recombinant DNA methodologies and is well known to those skilled in the art. Such methods are described in *Current Protocols in Molecular Biology* (John Wiley &  
20 Sons, Inc.), which is incorporated herein by reference.

Additionally, the preferred embodiments include a biologically active peptide derived from the mutein described herein. Such a peptide will contain at least one of the substitutions described and the mutein will possess biological activity. The peptide may be produced by any and all means known to those skilled in the art, examples of which included  
25 but are not limited to enzymatic digestion, chemical synthesis or recombinant DNA methodologies.

It is established in the art that fragments of peptides of certain fibroblast growth factors are biologically active. See for example, Baird et al., *Proc. Natl. Acad. Sci (USA)* 85:2324-2328 (1988), and *J. Cell. Phys. Suppl.* 5:101-106 (1987). Therefore, the selection of  
30 fragments or peptides of the mutein is based on criteria known in the art. For example, it is known that dipeptidyl peptidase IV (DPP-IV) is a serine type protease involved in inactivation of neuropeptides, endocrine peptides, and cytokines (Damme et al. *Chem. Immunol.* 72: 42-56, (1999)). The N-terminus of FGF-21 (HisProIlePro) contains two dipeptides that could potentially be substrates to DPP-IV, resulting in a fragment of FGF-21  
35 truncated at the N-terminus by 4 amino acids. Unexpectedly, this fragment of wild-type FGF-21 has been demonstrated to retain biological activity (Table 1), thus, muteins of the

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5 present invention truncated at the N-terminus by up to 4 amino acids, is an embodiment of the present invention.

The present invention also encompasses polynucleotides encoding the above-described muteins that may be in the form of RNA or in the form of DNA, which DNA includes cDNA, genomic DNA, and synthetic DNA. The DNA may be double-stranded  
10 or single-stranded. The coding sequences that encode the muteins of the present invention may vary as a result of the redundancy or degeneracy of the genetic code.

The polynucleotides that encode for the muteins of the present invention may include the following: only the coding sequence for the mutein, the coding sequence for the mutein and additional coding sequence such as a functional polypeptide, or a leader or  
15 secretory sequence or a pro-protein sequence; the coding sequence for the mutein and non-coding sequence, such as introns or non-coding sequence 5' and/or 3' of the coding sequence for the mutein. Thus the term "polynucleotide encoding a mutein" encompasses a polynucleotide that may include not only coding sequence for the mutein but also a polynucleotide, which includes additional coding and/or non-coding sequence.

20 The present invention further relates to variants of the described polynucleotides that encode for fragments, analogs and derivatives of the polypeptide that contain the indicated substitutions. The variant of the polynucleotide may be a naturally occurring allelic variant of the human FGF-21 sequence, a non-naturally occurring variant, or a truncated variant as described above. Thus, the present invention also includes  
25 polynucleotides encoding the muteins described above, as well as variants of such polynucleotides, which variants encode for a fragment, derivative or analog of the disclosed mutein. Such nucleotide variants include deletion variants, substitution variants, truncated variants, and addition or insertion variants as long as at least one of the indicated amino acid substitutions of the first or second embodiments is present.

30 The polynucleotides of the present invention will be expressed in hosts after the sequences have been operably linked to (i.e., positioned to ensure the functioning of) an expression control sequence. These expression vectors are typically replicable in the host organisms either as episomes or as an integral part of the host chromosomal DNA. Commonly, expression vectors will contain selection markers, e.g., tetracycline,  
35 neomycin, and dihydrofolate reductase, to permit detection of those cells transformed with the desired DNA sequences. The FGF-21 mutein can be expressed in mammalian

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5 cells, insect, yeast, bacterial or other cells under the control of appropriate promoters. Cell free translation systems can also be employed to produce such proteins using RNAs derived from DNA constructs of the present invention.

*E. coli* is a prokaryotic host useful particularly for cloning the polynucleotides of the present invention. Other microbial hosts suitable for use include *Bacillus subtilis*,  
10 *Salmonella typhimurium*, and various species of *Serratia*, *Pseudomonas*, *Streptococcus*, and *Staphylococcus*, although others may also be employed as a matter of choice. In these prokaryotic hosts, one can also make expression vectors, which will typically contain expression control sequences compatible with the host cell (e.g., an origin of replication). In addition, any of a number of well-known promoters may be present, such  
15 as the lactose promoter system, a tryptophan (trp) promoter system, a beta-lactamase promoter system, or a promoter system from phages lambda or T7. The promoters will typically control expression, optionally with an operator sequence, and have ribosome binding site sequences and the like, for initiating and completing transcription and translation.

20 One skilled in the art of expression of proteins will recognize that methionine or methionine-arginine sequence can be introduced at the N-terminus of the mature sequence (SEQ ID NO: 1) for expression in *E. coli* and are contemplated within the context of this invention. Thus, unless otherwise noted, muteins of the present invention expressed in *E. coli* have a methionine sequence introduced at the N-terminus.

25 Other microbes, such as yeast or fungi, may also be used for expression. *Pichia pastoris*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and *Pichia angusta* are examples of preferred yeast hosts, with suitable vectors having expression control sequences, such as promoters, including 3-phosphoglycerate kinase or other glycolytic enzymes, and an origin of replication, termination sequences and the like as desired.  
30 *Aspergillus niger*, *Trichoderma reesei*, and *Schizophyllum commune*, are examples of fungi hosts, although others may also be employed as a matter of choice.

Mammalian tissue cell culture may also be used to express and produce the polypeptides of the present invention. Eukaryotic cells are actually preferred, because a number of suitable host cell lines capable of secreting intact muteins have been developed  
35 in the art, and include the CHO cell lines, various COS cell lines, NS0 cells, Syrian

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5 Hamster Ovary cell lines, HeLa cells, or human embryonic kidney cell lines (*i.e.* HEK293, HEK293EBNA).

Expression vectors for these cells can include expression control sequences, such as an origin of replication, a promoter, an enhancer, and necessary processing information sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites, and  
10 transcriptional terminator sequences. Preferred expression control sequences are promoters derived from SV40, adenovirus, bovine papilloma virus, cytomegalovirus, Raus sarcoma virus, and the like. Preferred polyadenylation sites include sequences derived from SV40 and bovine growth hormone.

The vectors containing the polynucleotide sequences of interest (e.g., the muteins  
15 of FGF-21 and expression control sequences) can be transferred into the host cell by well-known methods, which vary depending on the type of cellular host. For example, calcium chloride transfection is commonly utilized for prokaryotic cells, whereas calcium phosphate treatment or electroporation may be used for other cellular hosts.

Various methods of protein purification may be employed and such methods are  
20 known in the art and described, for example, in Deutscher, *Methods in Enzymology* 182: 83-9 (1990) and Scopes, *Protein Purification: Principles and Practice*, Springer-Verlag, NY (1982). The purification step(s) selected will depend, for example, on the nature of the production process used for the muteins of FGF-21.

The FGF-21 mutein-containing compositions should be formulated and dosed in a  
25 fashion consistent with good medical practice, taking into account the clinical condition of the patient, the site of delivery of the FGF-21 mutein composition, the method of administration, the scheduling of administration, and other factors known to practitioners. The "therapeutically effective amount" of the FGF-21 mutein for purposes herein is thus determined by such considerations

30 The pharmaceutical compositions of the FGF-21 muteins and of the present invention may be administered by any means that achieve the generally intended purpose: to treat type 2 diabetes, obesity, metabolic syndrome, or critically ill patients. The term "parenteral" as used herein refers to modes of administration that include intravenous, intramuscular, intraperitoneal, intrasternal, subcutaneous, and intraarticular injection and  
35 infusion. The dosage administered will be dependent upon the age, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature



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5 of the effect desired. Compositions within the scope of the invention include all compositions wherein an FGF-21 mutein is present in an amount that is effective to achieve the desired medical effect for treatment type 2 diabetes, obesity, or metabolic syndrome. While individual needs may vary from one patient to another, the determination of the optimal ranges of effective amounts of all of the components is  
10 within the ability of the clinician of ordinary skill.

The muteins of FGF-21 of the present invention can be formulated according to known methods to prepare pharmaceutically useful compositions. A desired formulation would be one that is a stable lyophilized product that is reconstituted with an appropriate diluent or an aqueous solution of high purity with optional pharmaceutically acceptable  
15 carriers, preservatives, excipients or stabilizers [*Remington's Pharmaceutical Sciences* 16th edition (1980)]. The muteins of the present invention may be combined with a pharmaceutically acceptable buffer, and the pH adjusted to provide acceptable stability, and a pH acceptable for administration.

For parenteral administration, in one embodiment, the FGF-21 muteins are  
20 formulated generally by mixing one or more of them at the desired degree of purity, in a unit dosage injectable form (solution, suspension, or emulsion), with a pharmaceutically acceptable carrier, *i.e.*, one that is non-toxic to recipients at the dosages and concentrations employed and is compatible with other ingredients of the formulation. Preferably, one or more pharmaceutically acceptable anti-microbial agents may be added.  
25 Phenol, *m*-cresol, and benzyl alcohol are preferred pharmaceutically acceptable anti-microbial agents.

Optionally, one or more pharmaceutically acceptable salts may be added to adjust the ionic strength or tonicity. One or more excipients may be added to further adjust the isotonicity of the formulation. Glycerin, sodium chloride, and mannitol are examples of  
30 an isotonicity adjusting excipient.

Those skilled in the art can readily optimize pharmaceutically effective dosages and administration regimens for therapeutic compositions comprising an FGF-21 mutein, as determined by good medical practice and the clinical condition of the individual patient. A typical dose range for the FGF-21 muteins of the present invention will range  
35 from about 0.01 mg per day to about 1000 mg per day for an adult. Preferably, the dosage ranges from about 0.1 mg per day to about 100 mg per day, more preferably from

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5 about 1.0 mg/day to about 10 mg/day. Most preferably, the dosage is about 1-5 mg/day. The appropriate dose of an FGF-21 mutein administered will result in lowering blood glucose levels and increasing energy expenditure by faster and more efficient glucose utilization, and thus is useful for treating type 2 diabetes, obesity and metabolic syndrome.

10 In addition, because hyperglycemia and insulin resistance are common in critically ill patients given nutritional support, some ICUs administer insulin to treat excessive hyperglycemia in fed critically ill patients. In fact, recent studies document the use of exogenous insulin to maintain blood glucose at a level no higher than 110 mg per deciliter reduced morbidity and mortality among critically ill patients in the surgical  
15 intensive care unit, regardless of whether they had a history of diabetes (Van den Berghe, *et al. N Engl J Med.*, 345(19):1359, (2001)). Thus, muteins of FGF-21 of the present invention are uniquely suited to help restore metabolic stability in metabolically unstable critically ill patients. Muteins of FGF-21 are unique in that they stimulate glucose uptake and enhances insulin sensitivity but do not induce hypoglycemia.

20 In another aspect of the present invention, muteins of FGF-21 for use as a medicament for the treatment of type 2 diabetes, obesity, metabolic syndrome, or critically ill patients is contemplated.

Having now described the present invention in detail, the same will be more clearly understood by reference to the following examples, which are included herewith for  
25 purposes of illustration only and are not intended to be limiting of the invention.

All patents and publications referred to herein are expressly incorporated by reference.

5

Example 1Expression and Purification of FGF-21 Muteins in *E. coli*

The bacterial expression vector pET30a is used for bacterial expression in this example. (Novagen, Inc., Madison, Wisconsin). pET30a encodes kanamycin antibiotic resistance gene and contains a bacterial origin of replication ("ori"), a strong T7 phage-IPTG inducible promoter, a ribosome binding site ("RBS"), and suitable MCS with a number of unique restriction endonuclease cleavage sites. Conveniently for purification purpose, the vector can encode His- and S-tags for N-terminal peptide fusions, as well as, a C-terminal His-tag fusion. However, for purposes of the present invention, the cDNA encoding FGF-21 variants is inserted between restriction sites NdeI and BamHI, respectively, and the resulting construct does not take advantage of either of the described tags.

The nucleic acid sequence encoding the FGF-21 mutein, lacking the leader sequence but substituted with a methionine residue, is amplified from a cDNA clone using PCR oligonucleotide primers, which anneal to the 5' and 3' ends of the open reading frame. Additional nucleotides, containing recognition sites for restriction enzymes NdeI and BamHI, are added to the 5' and 3' sequences, respectively.

For cloning, the 5' forward and 3' reverse PCR primers have nucleotides corresponding or complementary to a portion of the coding sequence of the FGF-21 mutein-encoding nucleic acid according to methods known in the art. One of ordinary skill in the art would appreciate that the point in a polynucleotide sequence where primers begin can be varied.

The amplified nucleic acid fragments and the vector pET30a are digested with NdeI and BamHI restriction enzymes and the purified digested DNA fragments are then ligated together. Insertion of FGF-21 mutein-encoding DNA into the restricted pET30a vector places the FGF-21 mutein polypeptide coding region including its associated stop codon downstream from the IPTG-inducible promoter and in-frame with an initiating ATG codon. The associated stop codon, TAG, prevents translation of the six-histidine codons downstream of the insertion point.

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5       The ligation mixture is transformed into competent *E. coli* cells using standard procedures such as those described in *Current Protocols in Molecular Biology* (John Wiley & Sons, Inc.).

Transformation reactions are plated on LB/Kanamycin plates and after an overnight growth transformants are picked for plasmid preparations or lysed in situ for  
10   screening by PCR. Positive recombinant plasmids, containing desired FGF-21 variant inserts, are identified by restriction analysis followed by DNA sequence analysis. Those plasmids are subsequently used to transform expression strains and protein production.

*E. coli* strains BL21(DE3), BL21(DE3)STAR or BL21(DE3) RP, are used for expressing FGF-21 muteins. These strains, which are only some of many that are suitable  
15   for expressing FGF-21 muteins, are available commercially from Novagen, Inc., Invitrogen and Stratagen, respectively. Transformants are identified by their ability to grow on LB plates in the presence of kanamycin.

Clones containing the desired constructs are grown overnight (o/n) in liquid culture in LB media supplemented with kanamycin (30µg/ml). The o/n culture is used to  
20   inoculate a large culture, at a dilution of approximately 1:25 to 1:250. The cells are grown to an optical density of 0.6 ("OD600") at 600 nm. Isopropyl-b-D-thiogalactopyranoside ("IPTG") is then added to a final concentration of 1 mM to induce transcription from the lac repressor sensitive promoter, by inactivating the lacI repressor. Cells subsequently are incubated further for 3 to 12 hours. Cells are then harvested by  
25   centrifugation, pellets washed with 50 mM Tris buffer, pH 8.0 and stored at -20 °C until purification. The FGF-21 muteins are expressed in the insoluble fraction *i.e.* inclusion bodies (or granules) of *E. coli*. Although the expression level may vary from variant-to-variant, a typically observed level for the wild-type (WT) FGF-21 protein is 50 mg/L. The subsequent purification process starts with solubilization of the granules and  
30   refolding of the variants followed by four chromatographic steps.

To purify the FGF-21 muteins from *E. coli*, the granules are solubilized in 50 mM Tris, pH 9.0, 7M Urea and 1 mM DTT through a pH ramp to pH 11.0, at room temperature for 1 hour with stirring. The protein is then captured on a Q-Sepharose column using the same buffer described above, and eluted with a linear gradient of 0-400  
35   mM NaCl. The Q-Sepharose pool is then treated with 10 mM DTT, for two hours, at RT, to reduce all disulfide bonds. The pool is then diluted 10-fold so that the buffer

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5 concentration is as follows: 50 mM Tris, pH 9.0, 7 M Urea, 10 mM Cysteine, 1 mM DTT with a protein concentration of approximately 250-500 µg/ml. After another two-hour incubation under reducing conditions at RT, to obtain the protein in a free disulfide form, the pool is then dialyzed into 20 mM glycine, pH 9.0 for approximately 48 hours so that the correct disulfide bonds can be formed.

10 Reversed-phase HPLC chromatography, on a Vydac C18 column and 0.1% TFA/0-50% CH<sub>3</sub>CN as a mobile phase is used as an initial purification step. This column is used to concentrate FGF-21 or the FGF-21 muteins and removes contaminating endotoxin.

The following purification step is size exclusion chromatography on a Superdex 15 35/600 column performed in 1X PBS buffer, pH7.4. At this step FGF-21 muteins are ~95% pure. The last step involves MonoQ chromatography in 50 mM Tris, pH 8.0 and elution with a linear gradient of 0-300 mM NaCl, which usually yields > 97% pure protein.

The above described 4-column step purification scheme was used for all the FGF-21 20 muteins and produced stable preparations.

### Example 2

#### Expression and Purification of FGF-21 Muteins in HEK293EBNA Cells

Alternatively, FGF-21 muteins can be produced in a mammalian cell expression 25 system such as HEK293EBNA cells (EdgeBiosystems, Gaithersburg, MD). FGF-21 muteins are subcloned in the proprietary expression vector representing a modification of commercially available pEAK10, between *NheI* and *XbaI* restriction sites in the MCS. The cDNA sequence encoding mature FGF-21 is fused in frame with the Igκ leader sequence to enhance secretion of the desired product in the tissue culture media. The 30 expression is driven by the strong viral CMV promoter. HEK293EBNA cells are transiently transfected using a standard transfection reagent such as Fugene (Roche Diagnostics, Indianapolis, IN) and the appropriate amount of recombinant plasmid, either as a monolayer or suspension culture, at the adequate cell density. Cells are incubated at 37°C and 5 % CO<sub>2</sub>, in serum free media, and collections are made every day for 5 days. 35 Typically the expression level in the HEK293EBNA suspension culture is ~ 30 mg/L. The expression of human FGF-21 in mammalian cells yields the natural N-terminus

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5 sequence of HPIP, *i.e.* without a methionine residue at the N-terminus. It was discovered that enzymatically treating FGF-21 from HEK239EBNA cells with DPP-IV (porcine kidney, SIGMA St Louis) resulted in truncation of the N-terminus by four amino acids. When assayed in the mouse 3T3-L1 adipocyte assay (see Example 4), this truncated variant of FGF-21 stimulates glucose uptake at a comparable level to that of wild-type  
10 FGF-21 (Table 1).

### Example 3

#### Expression and Purification of FGF-21 Muteins in Yeast

Yet another expression system for production of FGF-21 muteins is yeast, such as  
15 *Pichia pastoris*, *Pichia methanolica* or *Saccharomyces cerevisiae*. For production in *Pichia pastoris* a commercially available system (Invitrogen, Carlsbad, CA) uses vectors with the powerful AOX1 (alcohol oxidase) promoters to drive high-level expression of recombinant proteins. Alternatively, vectors that use the promoter from the GAP gene (glyceraldehyde-3-phosphate dehydrogenase) are available for high level constitutive  
20 expression. The multi-copy *Pichia* expression vectors allows one to obtain strains with multiple copies of the gene of interest integrated into the genome. Increasing the number of copies of the gene of interest in a recombinant *Pichia* strain can increase protein expression levels. Yet another yeast expression system is *Saccharomyces cerevisiae*. Expression vectors contain the promoter and enhancer sequences from the GAL1 gene.  
25 The GAL1 promoter is one of the most widely used yeast promoters because of its strong transcriptional activity upon induction with galactose.

Analytical characterization (mass spectrum analyses) indicates that the FGF-21 expressed in *Pichia pastoris* is truncated (up to four amino acid removal [HisProIlePro] at the N-terminus, designated hereinafter as *des*-HPIP). When assayed in the mouse 3T3-L1  
30 adipocyte assay (see Example 4), this truncated variant of FGF-21 stimulates glucose uptake at the same level as wild-type FGF-21 (Table 1).

### Example 4

#### Glucose Uptake in Mouse 3T3-L1 Adipocytes

35 3T3-L1 cells are obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells are cultured in growth medium (GM) containing 10% iron-

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5 enriched fetal bovine serum in Dulbecco's modified Eagle's medium. For standard adipocyte differentiation, two days after cells reached confluency (referred as day 0), cells are exposed to differentiation medium (DM) containing 10% fetal bovine serum, 10  $\mu\text{g/ml}$  of insulin, 1  $\mu\text{M}$  dexamethasone, and 0.5  $\mu\text{M}$  isobutylmethylxanthine, for 48 h. Cells then are maintained in post differentiation medium containing 10% fetal bovine  
 10 serum, and 10  $\mu\text{g/ml}$  of insulin.

*Glucose Transport Assay*-- Hexose uptake, as assayed by the accumulation of 0.1 mM 2-deoxy-D- $^{14}\text{C}$ glucose, is measured as follows: 3T3-L1 adipocytes in 12-well plates are washed twice with KRP buffer (136 mM NaCl, 4.7 mM KCl, 10 mM  $\text{NaPO}_4$ , 0.9 mM  $\text{CaCl}_2$ , 0.9 mM  $\text{MgSO}_4$ , pH 7.4) warmed to 37 °C and containing 0.2% BSA,  
 15 incubated in Leibovitz's L-15 medium containing 0.2% BSA for 2 h at 37°C in room air, washed twice again with KRP containing, 0.2% BSA buffer, and incubated in KRP, 0.2% BSA buffer in the absence ( $\text{Me}_2\text{SO}$  only) or presence of wortmannin for 30 min at 37 °C in room air. Insulin is then added to a final concentration of 100 nM for 15 min, and the uptake of 2-deoxy-D- $^{14}\text{C}$ glucose is measured for the last 4 min. Nonspecific uptake,  
 20 measured in the presence of 10  $\mu\text{M}$  cytochalasin B, is subtracted from all values. Protein concentrations are determined with the Pierce bicinchoninic acid assay. Uptake is measured routinely in triplicate or quadruplicate for each experiment.

*In vitro* potency is normalized to the *in vitro* activity of wild-type FGF-21, which is given a designation of 1.0 and used as a positive control. The *in vitro* potency of  
 25 muteins of FGF-21 of the present invention is compared to wild-type FGF-21 in Table 1. As indicated in Table 1, the muteins of the present invention maintained biological potency to various degrees compare to wild-type FGF-21.

Table 1

FGF-21 Mutein	<i>Expression Expression System</i>	<i>In vitro Potency</i>
Wild-type	E. coli	1.0
Truncated Wild-type*	Yeast	0.9
Truncated Wild-Type**	HEK293EBNA	1.3

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Wild-type	HEK293EBNA	0.7
R77E	HEK293EBNA	1.1
L139E	E. coli	0.1
L146E	E. coli	0.8
Q156E	E. coli	0.6
S163E	E. coli	1.3
I152E/S163E	E. coli	0.9
A145E	E. coli	0.5
I152E	E. coli	1.2
L118C/A134C	E. coli	0.4
<i>des</i> -HPIP-L118C/A134C	Yeast	0.3

5 \*truncated by 4 amino acids at the N-terminus, *i.e.* *des*-HPIP

\*\*enzymatically truncated by 4-amino acids at the N-terminus by DPP-IV, *i.e.* *des*-HPIP

#### Example 5

##### *Ob/ob* Mouse Model

10 A study in an obesity model using male *ob/ob* mice was done to monitor plasma glucose levels and triglyceride levels after treatment with FGF-21, compared to vehicle and insulin control groups. The test groups of male *ob/ob* mice (7 weeks old) were injected with vehicle alone (0.9% NaCl), or FGF-21 mutein (0.125 mg/kg) subcutaneously (0.1 mL, once daily) for seven days. Blood was collected by tail clip

15 bleeding on day 7, one hour after the last compound injection and plasma glucose levels were measured using a standard protocol. The ability of the FGF-21 muteins to lower plasma glucose levels as compared to the vehicle control is shown in Table 2. The data in Table 2 indicates that muteins of the present invention lowered plasma glucose levels as compared to vehicle control. The ability of the FGF-21 muteins to lower triglyceride

20 levels as compared to the vehicle control is shown in Table 3.



5

**Table 2**

<b>FGF-21 Mutein</b>	<b><i>Plasma Glucose levels as % of Control</i></b>
Wild-type	60%
R77E	63%
Q156E	65%
S163E	60%
A145E	81%
I152E	82%
G161E	78%
L118C-A134C	80%

**Table 3**

<b>FGF-21 Mutein</b>	<b><i>Triglyceride Levels (mg/dL)</i></b>
Experiment #1	
Vehicle Control	200
Wild-type	145
R77E	125
Experiment #2	
Vehicle Control	165
Wild-type	90
Q156E	80
S163E	70
Experiment #3	
Vehicle Control	100
Wild-type	75
A145E	70
I152E	60
G161E	70
L118C-A134C	75

5

Example 6Pharmaceutical Stability of FGF-21 Muteins

The stability of the FGF-21 muteins of the present invention was analyzed under simulated physiological and pharmaceutical formulation conditions. To simulate physiological conditions, the mutein was analyzed for stability in PBS at room temperature (RT) at a target protein concentration of 10 mg/ml, pH 7.4. Solubility/physical stability of the muteins in PBS is considered satisfactory if recovery of protein following preparation resulted in >90% recovery at RT as determined by size-exclusion and/or reversed-phase chromatography. The muteins of the present invention indicated in Tables 4 and 5 meet this criteria.

It is anticipated that pharmaceutical formulation of a mutein of the present invention will likely be a preserved multi-use formulation, thus, compatibility with a common preservative was analyzed. To test for formulation compatibility, a preservative, *m*-cresol, (3 mg/mL final concentration, a concentration usually sufficient to meet European Pharmacopia B criteria for preservative effectiveness under neutral pH conditions), was added at room temperature to a solution containing the mutein at approximately 10mg/ml in PBS, pH 7.4. Physical stability in the presence of preservative was initially assessed by determining protein recovery of the main chromatographic peak after reversed-phase and size exclusion chromatography at RT. Furthermore, the extent of aggregation as measured by DLS (dynamic light scattering) at 37°C is shown as the average diameter of particles in the presence of *m*-cresol after two hours, compared to wild-type FGF-21. A larger average diameter corresponds to an increased degree protein association and/or aggregation. The preservative compatibility (as a function average diameter of particulates) of the muteins of the first and second embodiments of the present invention compared to wild-type FGF-21 is shown in Table 4. All muteins were expressed in *E. coli*.

Muteins of the present invention that are stable in PBS and compatible with preservative are designated to have enhanced or improved pharmaceutical properties as compared to wild-type FGF-21. As shown in Table 4, the preferred muteins of the present invention that have enhanced pharmaceutical properties as compared to wild-type FGF-21 are L139E, A145E, L146E, I152E, Q156E, [I152E, S163E], S163E, Q54E, [L21C-L33C, L118C-A134C], L21C-L33C, A26C-K122C, and L118C-A134C.

5

Table 4

<b>FGF-21 Mutein</b>	<b>Average Particulate Diameter (nm)*</b>
<b>Experiment #1</b>	
Wild-type FGF-21	1356
Q54E	210
L139E	234
A145E	223
L146E	248
I152E	76
Q156E	353
I152E, S163E	179
S163E	154
<b>Experiment #2</b>	
Wild-type FGF-21	813
L21C, L33C, L118C, A134C	10
L21C-L33C	10
L118C-A134C	7
A26C-K122C	7

\*Average Particulate diameter represents a protein solution at a target conc. of 10 mg/ml, *m*-cresol at 3 mg/ml, after 2 hours incubation at 37 °C.

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What is Claimed is:

- 10        1. A mutein of human fibroblast growth factor 21 (FGF-21), or a biologically active peptide thereof, comprising the substitution of a charged and/or polar but uncharged amino acid for one or more of the following: glycine 42, glutamine 54, arginine 77, alanine 81, leucine 86, phenylalanine 88, lysine 122, histidine 125, arginine 126, proline 130, arginine 131, leucine 139, alanine 145, leucine 146, isoleucine 152, alanine 154, glutamine 156, glycine 161, serine 163, glycine 170, or serine 172, wherein the numbering of the amino acids is based on SEQ ID NO:1.
- 15        2. The mutein of Claim 1 wherein the negatively charged amino acid is selected from the group consisting of aspartate, glutamate, and non-naturally occurring analogs thereof.
- 20        3. The mutein of Claim 1 wherein the polar but uncharged amino acid is selected from the group consisting of serine, threonine, asparagine, glutamine, and non-naturally occurring analogs thereof.
- 25        4. The mutein of Claim 1, wherein said mutein is selected from the group consisting of Leu139Glu, Ala145Glu, Leu146Glu, Ile152Glu, Gln156Glu, Ser163Glu, Ile152Glu, Ser163Glu, and Gln54Glu.
- 30        5. The mutein of Claim 4 wherein said mutein is truncated at the N-terminus by up to 4 amino acids.
6. A polynucleotide encoding the mutein of Claim 1.
7. The polynucleotide of Claim 6, wherein said polynucleotide is DNA.
- 35        8. An expression vector containing the DNA of Claim 7.

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9. A host cell comprising the expression vector of Claim 8.

10. The host cell of Claim 9 wherein said host cell is a yeast cell.

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11. A process for producing a polypeptide comprising:

(a) expressing said polypeptide from the host cell of Claim 10, and;

(b) isolating said polypeptide.

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12. A pharmaceutical composition comprising a therapeutically effective amount of the FGF-21 mutein of Claim 1 and an acceptable pharmaceutical carrier, wherein said composition is useful for treating a patient exhibiting one or more of the indications from the group consisting of obesity, type 2 diabetes, insulin resistance, hyperinsulinemia, glucose intolerance, hyperglycemia, or metabolic syndrome

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13. A method for treating a patient comprising administering to said patient a therapeutically effective amount of the FGF-21 mutein of Claim 1, wherein said patient exhibits one or more of the indications from the group consisting of obesity, type 2 diabetes, insulin resistance, hyperinsulinemia, glucose intolerance, hyperglycemia, or metabolic syndrome.

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14. The method of Claim 13 wherein said patient exhibits type 2 diabetes.

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15. A mutein of human FGF-21, or a biologically active peptide thereof, comprising the substitution of a cysteine for two or more of the following: arginine 19, tyrosine 20, leucine 21, tyrosine 22, threonine 23, aspartate 24, aspartate 25, alanine 26, glutamine 27, glutamine 28, alanine 31, leucine 33, isoleucine 35, leucine 37, valine 41, glycine 42, glycine 43, glutamate 50, glutamine 54, leucine 58, valine 62, leucine 66, glycine 67, lysine 69, arginine 72, phenylalanine 73, glutamine 76, arginine 77, aspartate 79, glycine 80, alanine 81, leucine 82, glycine 84, serine 85, proline 90, alanine 92, serine 94, phenylalanine 95, leucine 100, aspartate 102, tyrosine 104, tyrosine 107, serine 109, glutamate 110, proline 115, histidine 117, leucine 118, proline 119, asparagine 121, lysine

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- 5 122, serine 123, proline 124, histidine 125, arginine 126, aspartate 127, alanine 129, proline 130, glycine 132, alanine 134, arginine 135, leucine 137, proline 138, or leucine 139, wherein the numbering of amino acids is based on SEQ ID NO:1.

- 10 16. The mutein of Claim 15, wherein said mutein is selected from the group consisting of Leu21Cys-Leu33Cys/Leu118Cys-Ala134Cys, Leu21Cys/Leu33Cys, Leu118Cys/Ala134Cys, or Ala26Cys/Lys122Cys.

17. The mutein of Claim 16 wherein said mutein is truncated at the N-terminus by up to 4 amino acids.

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18. The mutein of Claim 17 wherein said mutein is *des*-HPIP-Leu118Cys/Ala134Cys.

19. A polynucleotide encoding the mutein of Claim 15.

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20. The polynucleotide of Claim 19, wherein said polynucleotide is DNA.

21. An expression vector containing the DNA of Claim 20.

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22. A host cell comprising the expression vector of Claim 21.

23. The host cell of Claim 22 wherein said host cell is a yeast cell.

24. A process for producing a polypeptide comprising:

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- (a) expressing said polypeptide from the host cell of Claim 23, and;  
(b) isolating said polypeptide.

25. A pharmaceutical composition comprising a therapeutically effective amount of the FGF-21 mutein of Claim 15 and an acceptable pharmaceutical carrier, wherein said composition is useful for treating a patient exhibiting one or more of the indications from

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-30-

5 the group consisting of obesity, type 2 diabetes, insulin resistance, hyperinsulinemia, glucose intolerance, hyperglycemia, or metabolic syndrome.

26. A method for treating a patient comprising administering to said patient a therapeutically effective amount of the FGF-21 mutein of Claim 15, wherein said patient  
10 exhibits one or more of the indications from the group consisting of obesity, type 2 diabetes, insulin resistance, hyperinsulinemia, glucose intolerance, hyperglycemia, or metabolic syndrome.

27. The method of Claim 26 wherein said patient exhibits type 2 diabetes.

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28. A mutein of human FGF-21, or a biologically active peptide thereof, comprising the substitution of a charged and/or polar but uncharged amino acid for one or more of the amino acids at positions: glycine 42, glutamine 54, arginine 77, alanine 81, leucine 86, phenylalanine 88, lysine 122, histidine 125, arginine 126, proline 130,  
20 arginine 131, leucine 139, alanine 145, leucine 146, isoleucine 152; alanine 154; glutamine 156, glycine 161, serine 163, glycine 170, or serine 172; in combination with the substitution of a cysteine for two or more of the amino acid at positions: arginine 19, tyrosine 20, leucine 21, tyrosine 22, threonine 23, aspartate 24, aspartate 25, alanine 26, glutamine 27, leucine 28, alanine 31, leucine 33, isoleucine 35, leucine 37, valine 41,  
25 glycine 42, glycine 43, glutamate 50, glutamine 54, leucine 58, valine 62, leucine 66, glycine 67, lysine 69, arginine 72, phenylalanine 73, glutamine 76, arginine 77, aspartate 79, glycine 80, alanine 81, leucine 82, glycine 84, serine 85, proline 90, alanine 92, serine 94, phenylalanine 95, leucine 100, aspartate 102, tyrosine 104, tyrosine 107, serine 109, glutamate 110, proline 115, histidine 117, leucine 118, proline 119, asparagine 121, lysine  
30 122, serine 123, proline 124, histidine 125, arginine 126, aspartate 127, alanine 129, proline 130, glycine 132, alanine 134, arginine 135, leucine 137, proline 138, or leucine 139, wherein the numbering of amino acids is based on SEQ ID NO:1.

29. A polynucleotide encoding the mutein of Claim 28.

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30. The polynucleotide of Claim 29, wherein said polynucleotide is DNA.

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31. An expression vector containing the DNA of Claim 30.

32. A host cell comprising the expression vector of Claim 31.

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33. The host cell of Claim 32 wherein said host cell is a yeast cell.

34. A process for producing a polypeptide comprising:

- (a) expressing said polypeptide from the host cell of Claim 33, and;
- (b) isolating said polypeptide.

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35. A pharmaceutical composition comprising a therapeutically effective amount of the FGF-21 mutein of Claim 28 and an acceptable pharmaceutical carrier, wherein said composition is useful for treating a patient exhibiting one or more of the indications from the group consisting of obesity, type 2 diabetes, insulin resistance, hyperinsulinemia, glucose intolerance, hyperglycemia, or metabolic syndrome.

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36. A method for treating a patient comprising administering to said patient a therapeutically effective amount of the FGF-21 mutein of Claim 28, wherein said patient exhibits one or more of the indications from the group consisting of obesity, type 2 diabetes, insulin resistance, hyperinsulinemia, glucose intolerance, hyperglycemia, or metabolic syndrome.

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37. The method of Claim 36 wherein said patient exhibits type 2 diabetes.

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38. The mutein of Claim 28 wherein said mutein is truncated at the N-terminus by up to 4 amino acids.

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39. The use of the FGF-21 mutein of Claim 1 for the manufacture of a medicament for the treatment of one or more of the indications from the group consisting of obesity, type 2 diabetes, insulin resistance, hyperinsulinemia, glucose intolerance, hyperglycemia, or metabolic syndrome.



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5           40. The use of the FGF-21 mutein of Claim 15 for the manufacture of a  
medicament for the treatment of one or more of the indications from the group consisting  
of obesity, type 2 diabetes, insulin resistance, hyperinsulinemia, glucose intolerance,  
hyperglycemia, or metabolic syndrome.

10           41. The use of the FGF-21 mutein of Claim 28 for the manufacture of a  
medicament for the treatment of one or more of the indications from the group consisting  
of obesity, type 2 diabetes, insulin resistance, hyperinsulinemia, glucose intolerance,  
hyperglycemia, or metabolic syndrome.

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X-16280.ST25.txt  
SEQUENCE LISTING

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&lt;120&gt; Muteins of Fibroblast Growth Factor 21

&lt;130&gt; X-16280

&lt;160&gt; 2

&lt;170&gt; PatentIn version 3.2

&lt;210&gt; 1

&lt;211&gt; 181

&lt;212&gt; PRT

&lt;213&gt; human

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Leu Glu Ile Arg Glu Asp Gly Thr Val Gly Gly Ala Ala Asp Gln Ser  
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Pro Glu Ser Leu Leu Gln Leu Lys Ala Leu Lys Pro Gly Val Ile Gln  
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Ile Leu Gly Val Lys Thr Ser Arg Phe Leu Cys Gln Arg Pro Asp Gly  
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gtggggggcg ctgctgacca gagccccgaa agtctcctgc agctgaaagc cttgaagccg 180  
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gccctgtatg gatcgctcca ctttgaccct gaggcctgca gcttccggga gctgcttctt 300  
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tcc 543

## INTERNATIONAL SEARCH REPORT

Internat Application No  
PCT/US2004/037200

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/12 C07K14/50 A61K38/18 C12N1/19

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, Sequence Search, PAJ, WPI Data, EMBASE, CHEM ABS Data

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 03/011213 A (ELI LILLY AND COMPANY; GLASEBROOK, ANDREW, LAWRENCE; HAMMOND, LISA, JA) 13 February 2003 (2003-02-13) cited in the application claims page 6, line 28 - page 7, line 10; examples page 9, line 8 - line 26	1-41
A	WO 01/36640 A (CHIRON CORPORATION KYOTO UNIVERSITY) 25 May 2001 (2001-05-25) cited in the application page 8, line 22 - page 9 page 15, line 21 - page 18, line 12 page 17, line 26 - line 29 ----- -/--	1-41

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

## \* Special categories of cited documents :

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- \*Z\* document member of the same patent family

Date of the actual completion of the international search

24 March 2005

Date of mailing of the international search report

05/04/2005

Name and mailing address of the ISA

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# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US2004/037200

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>WO 03/059270 A (ELI LILLY AND COMPANY; HEUER, JOSEF, GEORG; KHARITONENKOV, ALEXEI) 24 July 2003 (2003-07-24) cited in the application page 8, line 12 - line 26 claims</p> <p>-----</p>	1-41

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US2004/037200

## Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
  
Although claims 13-14, 26-27 and 36-37 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

**INTERNATIONAL SEARCH REPORT**  
Information on patent family members

International Application No  
**PCT/US2004/037200**

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 03011213	A	13-02-2003	WO 03011213 A2 US 2004259780 A1	13-02-2003 23-12-2004
WO 0136640	A	25-05-2001	AU 1922101 A CA 2392103 A1 EP 1232264 A2 JP 2003516731 T WO 0136640 A2 US 2002164713 A1 US 6716626 B1 US 2005037457 A1 US 2004185494 A1	30-05-2001 25-05-2001 21-08-2002 20-05-2003 25-05-2001 07-11-2002 06-04-2004 17-02-2005 23-09-2004
WO 03059270	A	24-07-2003	AU 2003201810 A1 CA 2468610 A1 EP 1469880 A2 WO 03059270 A2	30-07-2003 24-07-2003 27-10-2004 24-07-2003